Biotechnology Letters (2006) 28: 425–430
DOI 10.1007/s10529-005-6073-3

Polycyclic aromatic hydrocarbon (PAH) degradation coupled to methanogenesis

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Received 27 October 2005; Revisions requested 16 November 2005; Revisions received 14 December 2005; Accepted 16 December 2005

Key words: 16S ribosomal RNA, anaerobic biodegradation, bromoethanesulfonic acid (BES), methanogenesis, polycyclic aromatic hydrocarbon (PAH)

Abstract

Baltimore Harbor (Baltimore, MD) sediments were utilized to initiate anaerobic enrichment cultures with polycyclic aromatic hydrocarbons (PAHs) in the absence of supplementary electron acceptors. Cultures amended with naphthalene and phenanthrene exhibited sustained, transferable degradation of the PAHs. Bromoethanesulfonic acid, a selective inhibitor of methanogenesis, inhibited the degradation of 200 μM naphthalene and phenanthrene; molecular characterization based on 16S rRNA sequences confirmed that methanogenic *Archaea* were eliminated, thus providing evidence that methanogenesis is involved in the degradation pathway.

Introduction

Methanogenesis is a terminal electron accepting process for the degradation of organic compounds where other electron acceptors such as O_2, nitrate, and sulfate are depleted. Simple biomolecules are easily converted to methane and CO_2 in methanogenic biodegradation (Schink 1997). Nonetheless, little information has been available on the methanogenic degradation of relatively complex hydrocarbons. Recently, methanogenesis was reported to be involved in the biodegradation of long-chain alkanes (Zengler *et al.* 1999, Anderson & Lovley 2000). However, there has been no report providing evidence that methanogenesis is involved in anaerobic biodegradation of polycyclic aromatic hydrocarbons (PAHs).

Sulfate reduction and nitrate reduction are terminal electron accepting processes for PAH degradation (Coates *et al.* 1996, Zhang & Young 1997, Rockne & Strand 1998, 2001). Further, our previous study showed that anaerobic cultures were capable of degrading PAHs in the absence of supplementary electron acceptors (Chang *et al.* 2001, 2005a). However, that study utilized sediment-containing cultures that might provide necessary electron acceptors for the microbial degradative activity. Consequently, the main goal of this present study is to confirm whether the degradation is coupled to methanogenic metabolism. In detail, bromoethanesulfonic acid (BES), a selective inhibitor of methanogenesis (Gunsalus *et al.* 1978, Harada *et al.* 2001), was added to actively PAH-degrading anaerobic cultures to see if there was any inhibitory effect. Methane production was monitored as well to indirectly evaluate methanogenic activity. Moreover, phylogenetic analysis based on 16S rRNA (rDNA) sequences was performed to verify the structure of microbial communities including methanogenic *Archaea*. Finally, the results presented herein may demonstrate the applicability of methanogenic metabolism for anaerobic PAH degradation.

Materials and methods

Enrichment cultures and monitoring

Enrichment cultures were initiated by anaerobically transferring Baltimore Harbor sediments (10% w/v) into sterile estuarine medium in an atmosphere that contained N_2 (75%), CO_2 (20%) and H_2 (5%) (Holoman *et al.* 1998; Chang *et al.* 2005a). The cultures were incubated with 200 μM naphthalene or phenanthrene without shaking in the dark at 30 °C. For the inhibitor studies, bromoethanesulfonic acid (BES), known to selectively inhibit methanogenic activity, was dissolved in deionized water, filter sterilized, and added to 3 mM. All enrichment cultures were made in triplicate. PAH concentration in the cultures and methane production in the headspace gas were monitored by using GC with flame ionization detection (Phelps *et al.* 1998, Chang *et al.* 2005a, b).

Creation of 16S rDNA library

Genomic DNA was isolated with the UltraClean Soil DNA Kit (MoBio Laboratories, Inc., Solana Beach, CA), by following the manufacturer's recommendation. Bacterial 16S rDNAs were amplified using polymerase chain reaction (PCR) from the mixed community of genomic DNAs. PCR amplification of bacterial 16S rDNAs was performed with Universal primers 519F (5′-CAG CA/CG CCG CGG TAA TA/TC-3′) and 1406R (5′-ACG GGC GGT GTG TA/GC-3′) (Chang *et al.* 2005a, b). *Archaeal* 16S rDNAs were amplified with *Alchaea*-specific primers 340F (5′-CCT ACG GGG CGC AC/GC AGG CC/GG C-3′) and 915R (5′-GTG CTC CCC CGC CAA TTC CT-3′) (Chang *et al.* 2005a, b). PCR conditions were as follows: an initial denaturation step of 1.5 min at 94 °C; 30 amplification cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and elongation (30 s at 72 °C); and a final extension step of 5 min at 72 °C. The PCR products were cloned in plasmids using the Invitrogen TA Cloning Kit (Invitrogen, Carlsbad, CA) and then plasmid libraries were transformed into *Escherichia coli* INVαF′ competent cells supplied with the Invitrogen TA Cloning Kit. Ninety-six clones for bacterial populations and 48 clones for *Archaeal* populations were randomly selected from colonies and grown overnight individually in Luria–Bertani (LB) medium with kanamycin (100 μg/ml).

Restriction fragment length polymorphism (RFLP) analysis

The partial 16S rDNA fragments were amplified directly from the overnight-grown LB cultures with the same, respective primers described above. PCR conditions are as follows: 1 cycle of 3 min at 95 °C; 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension step of 72 °C for 5 min. Accordingly, 96 bacterial and 48 *Archaeal* PCR products were generated. Then, those PCR products were digested separately with two restriction endonucleases, *Hae*III and *Hha*I (New England Biolabs, Inc., Beverly, MA) at 37 °C for 2 h. The digests were electrophoresed on a 3% Trevi-Gel (Trevi-Gen, Gaithersburg, MD) and visualized with ethidium bromide gel stain. Clones were categorized according to their distinct RFLPs. Note, in this study, RFLP type is used to refer to clones exhibiting a specific RFLP pattern.

Comparative sequence analysis

At least two representative clones for each unique RFLP pattern were sequenced for comparative phylogenetic analysis. Plasmid DNA was purified with the Qiagen Plasmid Mini Kit (Qiagen Inc., Valencia, CA). Approximately 900-bp segments were sequenced by Applied Biosystems DNA sequencer 3100 (Applied Biosystems, Foster City, CA) with primers M13REV (CAG GAA ACA GCT ATG ACC) and M13–21 (TGT AAA ACG ACG GCC AGT) at the DNA sequencing facility at the University of Maryland Biotechnology Institute. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (Altschul *et al.* 1990) and the Sequence_Match program of the Ribosomal Database Project II (Olsen *et al.* 1991). Moreover, the Chimera_Check program of the Ribosomal Database Project II was employed for chimera screening (Holoman *et al.* 1998). Sequences of the partial 16S rRNA clones were submitted to GenBank under accession numbers presented in Table 1.

Table 1. Phylogenetic affiliations of predominant RFLP types from naphthalene- or phenanthrene-degrading methanogenic cultures before and after BES addition based on 16S rRNA gene sequences.

RFLP type	NCBI accession no.	Closest phylogenetic relative (NCBI accession no.)	Sequence similarity (%)
U1[a]	AY261388	*Methanococcus vannielii* (M36507)	98
U2[a]	AY261389	Uncultured bacterium BURTON-35 (AF142853)	97
U5[a,]	AY261392	Unidentified eubacterium RFLP1 (AF058000)	98
U6[a]	AY261393	Uncultured bacterium (AY540497)	91
U7[a]	AY261394	Bacterium 2BP-6 (AF121886)	99
U8[a]	AY261395	Bacterium Phenol-1 (AF121884)	91
U10[a]	AY261396	*Acidaminobacter hydrogenoformans* (AF016691)	99
U11[a]	AY261397	Uncultured bacterium (AJ853626)	91
U17[a]	AY261398	*Desulfobulbus propionicus* (AY548789)	95
U19[a]	AY261399	Uncultured bacterium SJA-68 (AJ009475)	91
U20[a]	AY261400	Uncultured bacterium SHA-21 (AJ249103)	91
U27[a]	AY261401	Unidentified eubacterium RFLP15 (AF058004)	96
U30	AY871178	Uncultured bacterium RFLP U145 (AY871190)	97
U34	AY871179	Uncultured bacterium (AY799890)	96
U37[a]	AY261402	*Pseudomonas* cf. *pseudoalcaligenes* (AF181570)	99
U38[a]	AY261403	Uncultured *Actinomycete* SUBT-5 (AF361216)	98
U43[a]	AY261404	Uncultured bacterium PL-16B6 (AY570599)	92
U44[a]	AY261405	Uncultured bacterium U20II_21p1 (AY547927)	88
U46[a]	AY261406	Uncultured bacterium (AB186885)	89
U48[a]	AY261407	Uncultured bacterium SJA-118 (AJ009489)	97
U49[a]	AY261408	Benzene mineralizing consortium SB-34 (AF029049)	98
U51[a]	AY261409	Uncultured eubaterium WCHBI-54 (AF050582)	95
U55[a]	AY261410	Uncultured bacterium (AY540497)	93
U57[a]	AY261411	Uncultured bacterium (AB092910)	88
U60	AY871187	Uncultured *Spirochaetes* (AB074941)	96
U65[a]	AY261413	Toluene-degrading methanogenic consortium bacterium (AF423186)	90
U66	AY871188	*Desulfomicrobium* sp. MSL97 (AB110547)	99
U144	AY871189	Uncultured low G+C Gram-positive bacterium (AY280641)	95
A1[a]	AY261426	*Methanococcus vannielii* (M36507)	98
A4[a]	AY261427	*Methanococcus maripaludis* (AF005049)	97
A16[a]	AY261428	*Methanosarcina lacustris* (AY260431)	98
A23[a]	AY261429	*Methanosarcina mazei* (AY196685)	99

[a]RFLP types detected in naphthalene- or phenanthrene-degrading cultures before BES addition (Chang *et al*. 2005a).

Results

To investigate whether methanogenesis is associated with anaerobic biodegradation of PAH, we added an inhibitor of methanogenesis, bromoethanesulfonic acid (BES), to the actively degrading cultures, and then monitored the presence of methanogenic populations with molecular analysis. BES has been widely employed to selectively inhibit methanogenic activity (Gunsalus *et al*. 1978, Harada *et al*. 2001). Thus, after the degradation of 200 μM naphthalene and phenanthrene in the transferred cultures began, 3 mM BES was added on day 48 and 51, respectively. Figure 1a, b show that the addition of BES brought about the partial inhibition of both naphthalene and phenanthrene degradation. Note that methane production also stopped after the addition of BES, possibly indicating that methanogenesis had ceased (data not shown). This result suggests that methanogenic populations were involved in PAH degradation and further, methanogenesis was a terminal electron accepting process for the degradation.

To profile the microbial community structures of the naphthalene- and phenanthrene-degrading

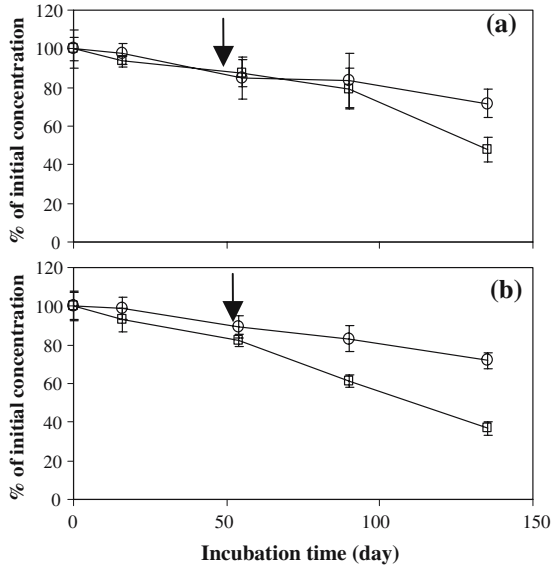

Fig. 1. Degradation of 200 μM naphthalene (a) and phenanthrene (b) in methanogenic enrichment cultures after the first transfer (10% [vol/vol]) with addition of BES (3mM) (→) on day 48 and 51, respectively. The results are the means of triplicates for active cultures (□) and inhibitor cultures (O). Relative concentration is the concentration measured relative to initial concentration (200 μM).

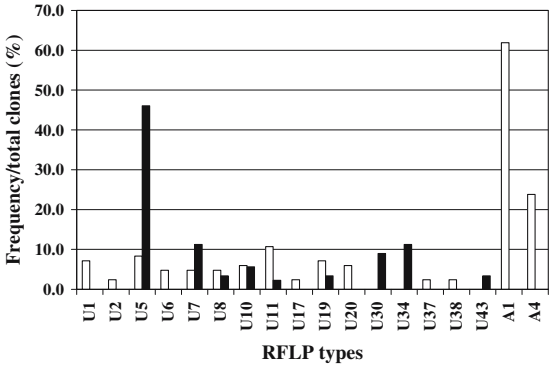

Fig. 2. Community profiles of 16S rDNA clones from naphthalene-degrading methanogenic enrichment cultures initiated with Baltimore Harbor sediments before (□) and after (■) the addition of BES. Note that RFLP types A1 and A4 were detected with *Archaeal* primers, whereas the other RFLP types were generated with Universal primers.

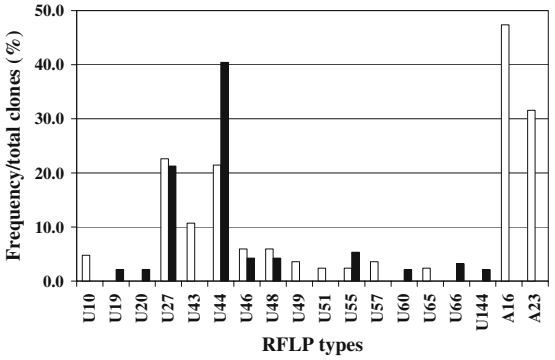

Fig. 3. Community profiles of 16S rDNA clones from phenanthrene-degrading methanogenic enrichment cultures initiated with Baltimore Harbor sediments before (□) and after (■) the addition of BES. Note that RFLP types A16 and A23 were detected with *Archaeal* primers, whereas the other RFLP types were generated with Universal primers.

cultures, we carried out 16S rRNA sequencing of isolated bacteria. One of the main reasons for performing this molecular analysis was to monitor methanogenic populations, the growth of which was supposed be stopped by BES. Therefore, we characterized the microbial communities before (Chang *et al.* 2005a) and after the addition of BES. Universal and *Archaeal* primers were employed for a PCR amplification of bacterial and *Archaeal* 16S rRNA genes, respectively. Most significantly, Figures 2 and 3 indicate that BES addition resulted in the disappearance of whole *Archaeal* populations (also see Table 1). To be specific, in the naphthalene cultures, methanogenic populations, RFLP types A1, A4, and U1, were not detected after BES addition (Figure 2). Phenanthrene cultures also demonstrated that RFLP types A16 and A23, which were closely related to *Methanosarcina* sp., disappeared after BES addition (Figure 3).

Figures 2 and 3 also show that BES addition caused the disappearance of some bacterial members of naphthalene- and phenanthrene-degrading communities. In detail, RFLP types U2, U6, U17, U20, U37, and U38 were not present in the naphthalene cultures (Figure 2), while RFLP types U10, U43, U49, U51, U57, and U65, were not detected in phenanthrene cultures (Figure 3). Moreover, several microorganisms that were not predominant in the absence of BES emerged after BES addition. Specifically, RFLP types U30, U34, and U43 were newly found with BES in the naphthalene cultures, whereas RFLP types U19, U20, U60, U66, and U144 were detected in the phenanthrene cultures. Table 1 exhibits that RFLP types U60 and U144 were closest to the phyla of *Spirochaetes*, and *Firmicutes*, respectively. Besides, RFLP type U66

was affiliated with a species of the *Deltaproteobacteria* class. The other RFLP types, U19, U20, U30, U34, and U43, were not close to any known phylogenetic subgroups.

Discussion

Methanogenesis is involved in the anaerobic degradation of easily degradable biomolecules such as carbohydrates and lipids (Zengler *et al*. 1999). However, only the degradation of monoaromatic hydrocarbons, alkenes, and alkanes involves methanogenesis (Zwolinski *et al*. 2000). The conclusion that methanogenesis is involved in anaerobic degradation cannot be drawn directly from the confirmation of degradation in the absence of any additional electron acceptors because in sediment-containing cultures, those electron acceptors could be provided by the sediments themselves. For instance, Holoman *et al*. (1998) reported that even without supplementary acceptors, not methanogenesis but sulfate-reduction was coupled to anaerobic tetrachlorobiphenyl degradation. Moreover, the abundance of sulfate-reducers in the PAH-degrading cultures revealed that a comparative phylogenetic analysis brought a question of whether methanogenesis was the predominant terminal electron accepting process for PAH degradation (Chang *et al*. 2005a). Therefore, in this study, a selective inhibitor of methanogenesis was utilized to confirm whether the degradation was associated with methanogenic *Archaea*.

Of most importance was that the molecular characterization confirmed that BES addition eliminated *Archaeal* methanogens (RFLP types U1, A1, A4, A16 and A23) (Table 1). This result, along with the finding that methane production was stopped, confirms that the inhibition on PAH degradation could be associated with the ceased methanogenesis by BES. Further, the molecular analysis revealed that not only methanogenic populations but also some bacterial ones vanished and further, certain populations likely emerged in the presence of BES. Indeed, complete degradation of relatively complex molecules by methanogenesis is known to require syntrophic cooperation among several different metabolic groups of bacteria such as hydrolytic fermentative bacteria, syntrophic acetogenic bacteria, and methanogenic bacteria (Ficker *et al*. 1999). Hence, if methanogens, which usually consume fermentative end products such as acetone and H_2 were not present, this accumulation could bring an inhibitory effect to the upper degradative process and possibly the initiation step, ultimately altering the structure of microbial communities, as the outcome here. It was also of interest that BES addition did not cause a complete inhibition of PAH degradation, as shown in Figure 1a, b, indicating that the species that initiated the degradation should be present even after BES addition. Thus, in detail, in naphthalene cultures, among RFLP types U5, U7, U8, U10, U11, and U19 and in phenanthrene cultures, among RFLP types U27, U44, U46, U48, and U55 member(s) could play that role in the degradative process.

In summary, this study is the first report, to our knowledge, demonstrating that methanogenic metabolism is coupled to anaerobic PAH degradation. The inhibited degradation, the ceased methane production, and the vanished methanogenic populations provided evidence for this conclusion. Methanogenic conditions are generally predominant around the source of contamination (Lovley 1997). Consequently, this research has important implications for enhancing the potential benefit of methanogenic metabolism to PAH remediation. Finally, efforts are currently under way to confirm the roles of each species and further isolate populations for anaerobic PAH degradation in the methanogenic enrichment communities.

Acknowledgement

This research was supported by National Science Foundation Faculty Early Career Award BES-9984285.

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